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Circulating Soluble RAGE Isoforms are Attenuated in Obese, Impaired Glucose Tolerant Individuals and are Associated with the Development of Type 2 Diabetes

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Running Head: Soluble RAGE isoforms and glucose tolerance status

Abstract

The soluble receptor for advanced glycation endproducts (sRAGE) may be protective against inflammation associated with obesity and type 2 diabetes (T2DM). The aim of this study was to determine the distribution of sRAGE isoforms, and whether sRAGE isoforms are associated with risk of T2DM development in subjects spanning the glucose tolerance continuum. In this retrospective analysis, circulating total sRAGE and endogenous secretory RAGE (esRAGE) were quantified via ELISA and cleaved RAGE (cRAGE) was calculated in 274 individuals stratified by glucose tolerance status (GTS) and obesity. Group differences were probed by ANOVA and multivariate ordinal logistic regression was used to test the association between sRAGE isoform concentrations and the proportional odds of developing diabetes, versus normal glucose tolerance (NGT) or impaired glucose tolerance (IGT). When stratified by GTS, total sRAGE, cRAGE, and esRAGE were all lower with IGT and T2DM, while the ratio of cRAGE to esRAGE (cRAGE:esRAGE) was only lower ($p<0.01$) with T2DM compared to NGT. When stratified by GTS and obesity, cRAGE:esRAGE was higher with obesity and lower with IGT ($p<0.0001$) compared to lean, NGT. In ordinal logistic regression models, greater total sRAGE (odds ratio: 0.91; $p<0.01$) and cRAGE (odds ratio: 0.84; $p<0.01$) were associated with lower proportional odds of developing T2DM. Reduced values of sRAGE isoforms observed with both obesity and IGT are independently associated with greater proportional odds of developing T2DM. The mechanisms by which each respective isoform contributes to obesity and insulin resistance may reveal novel treatment strategies for diabetes.

Key words: Receptor for Advanced Glycation End products, Type 2 Diabetes, obesity, insulin resistance, glucose tolerance

Abbreviations:
ADAM10 – A Disitigrin And Metalloproteinase 10
AGE – Advanced Glycation End products
CAD – Coronary Artery Disease
cRAGE – Cleaved Receptor for Advanced Glycation End products
esRAGE – Endogenous Secretory Receptor for Advanced Glycation End products
GDR – Glucose Disposal Rate
GTS – Glucose Tolerance Status
hnRNPA1 – Heterogeneous Nuclear Ribonuclear Protein A1
hs-CRP – High Sensitivity C-Reactive Protein
IGT – Impaired Glucose Tolerance
NGT – Normal Glucose Tolerance
RAGE – Receptor for Advanced Glycation End products
sRAGE – Soluble Receptor for Advanced Glycation End products
T2DM – Type 2 Diabetes
TRA2β - Transformer 2 Beta

Introduction

The study of advanced glycation end products (AGE) and their receptor (RAGE) has maintained scientific interest over the past several decades given evidence implicating them both as important contributors to the development, and progression of complications associated with diabetes (8, 33, 45). Initiation of inflammation and generation of reactive oxygen species as a consequence of RAGE activation is well documented (39). Despite numerous attempts, targeting RAGE directly as a therapeutic strategy has largely been unsuccessful (11). However, RAGE signaling can be interrupted, *in vivo*, by directed proteolytic cleavage of the RAGE ectodomain (cleaved RAGE: cRAGE) (16, 32), thus creating a soluble isoform of RAGE (sRAGE) that is released from the cell and appears into the circulation (32). In addition, alternative splicing of the RAGE gene at exon 9 produces a truncated c-terminus protein product (endogenous secretory RAGE: esRAGE) that is expelled from the cell via exocytosis

(56). This heterogeneous pool of solubilized receptors, collectively termed total sRAGE, serves to down-regulate the inflammatory response by absorbing excess RAGE ligands, thus attenuating cell membrane RAGE signaling. The production of soluble receptors, as a general concept, is regarded as a common feature of cytokine biology with significant implications for inflammatory disease progression and therapy. Thus, maintaining high levels of circulating sRAGE isoforms is apparently advantageous for the organism (14, 17, 48). This is exemplified, in-part, by data demonstrating sRAGE isoforms are decreased in inflammatory conditions such as type 2 diabetes mellitus (T2DM), coronary artery disease (CAD), and neurodegenerative diseases (14, 48, 54), while treatment with recombinant sRAGE (R-sRAGE), suppresses atherosclerosis and vascular dysfunction in animal models of diabetic CAD (34) .

Given this evidence, efforts have been made to establish the efficacy of sRAGE isoforms as biomarkers for diabetes and associated complications. However, existing clinical data are equivocal, possibly due to low sample size, lack of metabolic control measures and incomplete phenotyping. For example, several studies have demonstrated no difference or even elevated total sRAGE levels in T2DM compared to BMI-matched controls with no relationship to basic measures of insulin sensitivity such as HOMA-IR (4, 18). Alternatively, attenuated total sRAGE has been independently reported with obesity, pre-diabetes, and T2DM (5, 13, 40), and low total sRAGE was associated with greater risk of developing T2DM and cardiovascular mortality of non-diabetic individuals (40).

What these prior studies lack are normative values of sRAGE isoforms derived from a population of young, lean, and physically active adults, which is generally

regarded as the ideal state of human health. Further, no studies have yet to examine the independent effects of body composition or obesity on all sRAGE isoforms, nor have sRAGE isoforms been comprehensively examined across the glucose tolerance continuum, which underlies the natural history of T2DM. In addition, the relationships between sRAGE isoforms and insulin sensitivity remains ambiguous, potentially due to reliance on fasting indices of insulin sensitivity, such as HOMA-IR. Finally, cRAGE and esRAGE data are seldom reported together and the ratio of cRAGE to esRAGE (cRAGE:esRAGE) has yet to be explored as a potential index for insulin resistance or risk of developing T2DM. The latter may be particularly insightful given the mechanistic differences by which cRAGE and esRAGE are generated *in vivo*. Therefore, our aim was to characterize total sRAGE, cRAGE, esRAGE and cRAGE:esRAGE in a young, lean healthy reference group, as well as individuals stratified according to glucose tolerance status (GTS), obesity or both. We hypothesized that sRAGE isoforms would be reduced with impaired glucose tolerance (IGT) and T2DM and further reduced in the presence of obesity in comparison to a lean healthy reference group. Further, we assessed whether the circulating concentrations of sRAGE isoforms were associated with greater odds of developing T2DM.

Material and Methods

Study Design and Subjects

This data set examines 274 individuals from whom we have quantified circulating sRAGE isoform concentrations. Demographic and clinical data from some subjects participating in this work have been published (25, 31, 41, 42, 53). However, this is the first reporting of the sRAGE data in these subjects. Our intent was to examine sRAGE

isoforms and insulin sensitivity in a population of overweight and obese subjects that spanned the glucose tolerance continuum (NGT, IGT, T2DM), and directly contrast these observations with a group of young, lean healthy controls (LHC), who performed at least 120 minutes of moderate intensity physical activity per week. We interpret the LHC group to represent an optimal state of health and thus provide a benchmark of "normal" sRAGE isoform concentrations. Potential participants underwent medical screening to determine their eligibility for the study, which included a medical history assessment, electrocardiogram, and blood chemistry screening. Evidence of prior or current chronic pulmonary, hepatic, renal, gastrointestinal, or hematological disease, weight loss (>2 kg within 6 months), smoking, and contraindication to an exercise test were used as exclusion criteria. Blood glucose following a 2-hour oral glucose tolerance test (OGTT) was used to stratify subjects by GTS according to the American Diabetes Association (ADA) (2). However, T2DM stratification relied on ADA criteria, prior clinical diagnosis, or use of prescription anti-diabetic medication. Body mass index (BMI) was used to stratify subjects by obesity status (lean < 25 kg/m^2, overweight 25 – 29 kg/m^2, or obese > 29 kg/m^2). Subjects were recruited by newspaper/radio advertisement from the local municipal areas in Chicago, Illinois, Cleveland, Ohio, USA and Copenhagen, Denmark. All subjects provided oral and written informed consent prior to participation, and the methods were approved by local ethics committees at all locations (Institutional Review Boards of the University of Illinois at Chicago and Cleveland Clinic and the Scientific Ethics Committee of the Capital Region of Denmark).

Pre-Test Control Period

Tests took place in the Clinical Research Units of the University of Illinois at Chicago and Cleveland Clinic, and at the Clinical Research Laboratory of the Centre of Inflammation and Metabolism, Rigshospitalet, Denmark. Subjects being treated with anti-diabetic drugs withheld their medications for at least 24 hours prior to metabolic testing. Diet and physical activity records were taken in an outpatient setting and all subjects were instructed to abstain from consuming alcohol 48 hours prior to their visit and not to consume caffeine within 24 hours of their visit. Subjects also abstained from structured exercise for at least 24 hours prior to metabolic testing.

Clinical Procedures

Height and weight were measured using standard techniques. Whole body adiposity was estimated using dual-energy x-ray absorptiometry (Lunar iDXA, GE Healthcare, Madison, WI, USA). Subjects performed an incremental treadmill exercise test to determine their maximal oxygen consumption (VO_{2max}) as described previously (43). The VO_{2max} test was conducted at least 48-hours prior to subsequent metabolic assessments. On a separate day, following an 8-10 hour overnight fast, subjects came to the laboratory and an antecubital venous cannula was placed for baseline blood collection. Subjects ingested 75 g of anhydrous glucose dissolved in 300 mL water (standard OGTT). Following glucose ingestion, regular venous blood samples were collected for 2 hours. Blood was centrifuged at 2000 g for 15 min at room temperature and respective serum/plasma was stored at -80^{o}C until analysis. In addition, insulin sensitivity was measured in 80 subjects via hyperinsulinemic (40mU/m^2/min)-euglycemic (5 mmol/L) clamp. The methods of the hyperinsulinemic-euglycemic clamp were described previously (31, 53).

Blood Analyses

Glucose concentrations were measured using a bed-side analyzer (YSI Stat, Yellow Springs, USA; ABL, Radiometer, Denmark); insulin concentrations were determined by electrochemiluminescence immunoassay (E-modular; Roche, Switzerland) and radioimmunoassay (Millipore, Billerica, MA, USA); glycated hemoglobin (HbA_{1c}) levels were determined by high performance liquid chromatography (HPLC) (Tosoh G7 analyzer; San Francisco, CA, USA). High sensitive C-reactive protein (hs-CRP) was determined via ELISA (Alpha Diagnostics International, San Antonio, TX, USA). Total sRAGE concentrations were measured in plasma samples by commercial ELISA (R&D Systems Inc., Minneapolis, MN, USA) as per the manufacturer's protocol. This measure of total human sRAGE levels includes both the cleaved (cRAGE) and spliced variants (esRAGE). A monoclonal antibody raised against the N-terminal of the extracellular domain of RAGE, comprising amino acids 24-344, was used to detect the sRAGE in the sample (R&D Systems Inc.). Plasma esRAGE concentrations were measured separately by commercial ELISA (As One International, Mountain View, CA, USA) as per the manufacturer's protocol. A monoclonal antibody raised against human esRAGE, recognizing amino acids 332-347 was used to detect esRAGE in the sample (B-Bridge International). Plasma cRAGE concentrations were then determined by subtracting esRAGE from total sRAGE as previously described (47, 55). The sRAGE ratio (cRAGE:esRAGE) was derived by the quotient of cRAGE to esRAGE and expressed in arbitrary units. All samples were analyzed in duplicate.

Statistics

All data was tested for normality using Shapiro-Wilk's test. Parametric or non-parametric statistical tests were applied accordingly. Subject characteristics for each group were compared using a one-way ANOVA. One-way ANOVA was also used to compare mean sRAGE isoform data between groups. The effects of obesity (lean, overweight, obese) and glucose tolerance status (NGT, IGT, T2DM) on sRAGE isoforms were determined via two-way ANOVA. Bonferroni/Dunn post hoc tests were used for multiple comparisons when appropriate. Multivariate ordinal regression modeling was used to determine if sRAGE isoforms could predict risk of diabetes progression using stratification by glucose tolerance status and adjustment for age, race and obesity (proportional odds model) (52). Caucasian was used as the reference for race, and lean was used as the reference for obesity status. Total sRAGE, esRAGE, cRAGE and cRAGE:esRAGE were used to construct models. The values for total sRAGE, cRAGE and esRAGE were multiplied by 100 before entering them into the models. To avoid co-linearity, we did not generate a stepwise model that included all sRAGE measures in the model. Homogeneity of the odds ratios was confirmed for all variables prior to performing ordinal regression. Bivariate correlation analyses were performed using Pearson or Spearman correlation coefficients. SPSS v24 (IBM, Armonk, NY, USA) and SAS (Cary, NC, USA) were used to perform statistical analyses. $p < 0.05$ was considered significant and data are presented as mean ± SD.

Results

Subject Characteristics

Table 1 shows subject characteristics stratified by GTS. Markers of glycemic control (HbA_{1c}, 2-h OGTT glucose and fasting glucose) were progressively increased

across the glucose tolerance continuum. The IGT and T2DM groups were of similar age, BMI, and fitness level (VO_{2Max}) (Table 1; $p>0.05$). By design, compared to the IGT and T2DM groups, the NGT group was younger, leaner (BMI), more fit (VO_{2Max}) and had superior glycemic control apart from 2-h OGTT glucose iAUC, which was not different from T2DM (Table 1). Further details of subject characteristics including gender and race frequencies in each group are provided in Table 2.

sRAGE Isoforms are Attenuated with Impaired Glucose Tolerance

When stratified by GTS, NGT individuals had 33% (SD 37%) greater total sRAGE compared to IGT individuals ($p<0.05$) and 31% (SD 29%) greater total sRAGE compared to T2DM individuals ($p<0.05$; Figure 1A). cRAGE and esRAGE, which comprise total sRAGE, were lower to a similar extent in IGT and T2DM compared to NGT individuals ($p<0.05$; Figure 1B and C). However, cRAGE:esRAGE was only lower in T2DM compared to NGT subjects pointing to a disproportionate lack of cRAGE in T2DM individuals ($p<0.05$; Figure 1D). This observation is significant considering that cRAGE made up 63% (SD 12.5%) of total sRAGE in subjects with T2DM.

Increased circulating sRAGE Isoforms are associated with reduced proportional odds of developing diabetes

We had hypothesized that reduced sRAGE isoforms may underlie the natural history of T2DM according to progression across the glucose tolerance continuum. Using ordinal logistic regression analysis (Table 3), total sRAGE (Model 1), cRAGE (Model 2), esRAGE (Model 3), and cRAGE:esRAGE (Model 4) were combined with other independent variables (age, race, obesity) to form each respective model. As expected, and shown previously, both age and race were associated with greater

proportional odds (Table 3) for the development of T2DM (19). For total sRAGE, cRAGE, and cRAGE:esRAGE, each were independently associated with the proportional odds for progression across the glucose tolerance continuum to T2DM, whereas esRAGE was not. A 100 pg/mL increase in total sRAGE was associated with a 9% reduction in the proportional odds of developing T2DM, whereby the same increase in cRAGE was associated with a 16% reduction (Table 3). Additionally, every 1 unit increase in cRAGE:esRAGE predicted a 26% decreased risk of diabetes progression (Table 3). The model demonstrating the greatest reduction in proportional odds was Model 2 that included cRAGE isoforms (C-Statistic 0.805; Table 3).

Relationships with sRAGE isoforms and Metabolic Variables

Bivariate correlation analyses between sRAGE variables and metabolic variables are presented in Table 4. Total sRAGE, cRAGE, and esRAGE negatively correlated with BMI and percent body fat with esRAGE having the strongest relationships between both variables. In addition, all sRAGE variables were positively correlated with cardiorespiratory fitness (VO_{2Max}). Positive correlations between cRAGE:esRAGE, VO_{2Max} and BMI again demonstrate that the proportion of cRAGE and esRAGE isoforms, rather than just the independent quantity of each, is related to fitness level, and body weight status.

Apart from 2-h OGTT iAUC, total sRAGE and cRAGE negatively correlated with clinical markers of glycemic control (2-h OGTT glucose, HbA_{1c}, fasting glucose, fasting insulin, and HOMA-IR). On the other hand, esRAGE negatively correlated with 2-h OGTT iAUC, HbA_{1c}, and fasting glucose whereas sRAGE ratio positively correlated with 2-h OGTT iAUC, and negatively correlated with 2-h OGTT glucose, fasting glucose and

HOMA-IR. Finally, total sRAGE, esRAGE, and cRAGE all positively correlated with Matsuda index; however, the strongest associations with insulin sensitivity were found between clamp-derived glucose disposal rate (GDR) and total sRAGE (rho=0.472, p<0.001), cRAGE (rho=0.343, p=0.003), and esRAGE (rho=0.594, p <0.001). GDR also negatively correlated with cRAGE:esRAGE (rho=-0.276, p=0.018).

sRAGE Isoforms are Reduced with Worsening Obesity Status

Because the glucose tolerance groups were heterogeneous with regard to obesity, we further stratified by obesity status to isolate the sRAGE phenotype of lean NGT individuals. Because of low sample size in the overweight subgrouping, the IGT group was combined with T2DM (IGT-T2DM) and overweight was combined with obese (Overweight-Obese) (Figure 2). Using a 2-way (glucose tolerance x obesity) ANOVA, obesity status displayed a group effect for esRAGE (p=0.001) and cRAGE:esRAGE (p<0.0001). A group effect was also seen for GTS on total sRAGE (p<0.0001), cRAGE (p<0.0001), esRAGE (p=0.026), and cRAGE:esRAGE (p<0.0001), and an interaction effect was observed for total sRAGE (p=0.002), cRAGE (p=0.001), esRAGE (p=0.048), and cRAGE:esRAGE (p=0.032).

Lean, NGT individuals displayed the highest concentration of total sRAGE (Figure 2A), cRAGE (Figure 2B), and esRAGE (Figure 2C), compared to all other subgroups. The largest deviation from lean, NGT individuals when examining cRAGE was found in Lean, IGT-T2DM (61%, SD 16%). However, the largest deviation of esRAGE from lean, NGT individuals was found in Overweight-Obese, IGT-T2DM individuals (36%, SD 36%). Comparison of cRAGE:esRAGE between groups revealed the largest ratio exists in the Overweight-Obese, NGT group (Figure 2D). This increase

in cRAGE:esRAGE ratio indicates a preferential decrease in esRAGE related to worsening obesity status. Full analyses of the individual group stratifications and alternative sub groupings were performed and statistically interrogated via ANOVA. However, these analyses did not offer any insight beyond the results presented here.

We also analyzed the concentration of sRAGE isoforms across obesity status alone by stratifying individuals into lean, overweight or obese groups. Individuals who were overweight or obese had similar concentrations of sRAGE isoforms and 24-35% lower concentrations of sRAGE isoforms compared to lean individuals ($p<0.05$). Being that the NGT group was significantly younger than the IGT and T2DM groups (Table 2). Lastly, we examined the effect of age on sRAGE isoforms by stratifying individuals into young (18-35 y), middle-aged (36-64 y), and older (≥65 y) groups. Concentration of sRAGE isoforms were similar between middle-aged and older individuals but were 25-45% lower compared to young individuals ($p<0.05$). Interestingly, older individuals had a lower cRAGE:esRAGE ratio compared to both young and middle-aged individuals ($p<0.05$). Given this analysis demonstrated a significant effect of age on sRAGE isoforms, we examined the effect of GTS on sRAGE measures while co-varying for age as a continuous variable. The results of this analysis eliminated all significant effects of GTS on esRAGE and cRAGE:esRAGE concentration ($p>0.05$). In addition, the difference between total sRAGE and cRAGE in NGT compared to IGT groups that exist in Figure 1 were also resolved. However, even after controlling for age, individuals with T2DM still possess significantly lower total sRAGE and cRAGE compared to NGT individuals. All sRAGE isoforms were also negatively correlated with age (Table 4).

Discussion

To our knowledge, the current study is the first to report circulating concentrations of both major sRAGE isoforms (cRAGE and esRAGE) in the context of obesity and T2DM. Our primary finding was that lean, NGT individuals possessed the greatest concentration of sRAGE isoforms compared to states of obesity, IGT, T2DM or both. These findings are in accord with previous reports of lower sRAGE with obesity (5, 13, 18) and impaired glucose tolerance (3, 12, 22, 46). Importantly, we also demonstrate for the first time that reduced circulating concentrations of sRAGE isoforms are associated with greater proportional odds for the development of T2DM.

To this end, we developed ordinal logistic regression models using the sRAGE isoforms and cRAGE:esRAGE as independent variables to determine the proportional odds ratio of progression across the glucose tolerance continuum to T2DM. GTS is interpreted as having set thresholds along a range of possible outcomes according to American Diabetes Association criteria for the diagnosis of T2DM, thus meeting the assumption needed for ordinal regression (52). The application of this type of statistical model allows for hypothesizing movement along a known continuum (using proportional odds) without longitudinal follow-up. Importantly, our analyses revealed that a 100 pg/mL increase in total sRAGE and cRAGE resulted in a marked risk reduction for progression across the glucose tolerance continuum. For calibration, 100 pg/mL represents 12% of the cRAGE concentration in lean NGT individuals. Given our regression model, the lower cRAGE observed in IGT subjects (276 pg/mL) equates to ~44% increased proportional odds of progression towards T2DM. Our sample size was relatively small and our sampling was cross-sectional so these data must be interpreted with caution. However, Selvin et al. reported similar findings in a sample of 1,200

individuals without T2DM, whereby those in the lowest quartile of total sRAGE concentration had an increased risk of developing T2DM 18 years later (hazard ratio 1.64; 95% CI: 1.10-2.44) (40). Further, the relationships between modulation of sRAGE and health outcomes have been reported, whereby increased sRAGE, following a 12-wk aerobic exercise intervention, was associated with reduced C-reactive protein and improved aerobic fitness (9). Given the financial and time burden for longitudinal studies such as the latter, the application of ordinal regression models has merit for identification and characterization of novel targets such as sRAGE isoforms. Here, we expand on previous observations by demonstrating cRAGE as the isoform with the greatest ability to predict risk of progression across the glucose tolerance continuum whereas esRAGE did not. These data suggest dichotomous roles for cRAGE and esRAGE isoforms and their relevance to T2DM.

In line with this notion, we provide novel evidence of a disproportionate loss of cRAGE and esRAGE in the case of T2DM and obesity respectively. Although both cRAGE and esRAGE were significantly lower in IGT and T2DM compared to NGT individuals, only the T2DM group possessed a significantly lower cRAGE:esRAGE ratio. Additionally, when examining the effects of obesity and GTS on sRAGE measures, there was a significant effect of GTS on cRAGE:esRAGE ratio whereby impaired glucose tolerance tended to result in a lower ratio, implying a preferential loss of cRAGE (Figure 2). The lean, IGT-T2DM group stratification also possessed the lowest concentration of cRAGE compared to all other perturbations. Additionally, cRAGE correlated with 2h OGTT glucose and HOMA-IR whereas esRAGE did not. Collectively, these data suggest that loss of cRAGE is strongly influenced by IGT and T2DM.

The observed cRAGE phenotype may be mediated by a preferential attenuation of cRAGE-producing mechanisms with IGT and T2DM, specifically the proteolytic cleavage of the RAGE ectodomain via the enzyme A Disintigrin and Metalloproteinase 10 (ADAM10) or other matrix metalloproteinases (16, 32, 37). ADAM10 is the primary enzyme responsible for cRAGE production (37). Retinoic acid receptor beta (RARβ) positively regulates ADAM10 transcription by binding to its promoter site (28, 49). Deacetylation of RARβ, is necessary for this action and is mediated by the deacetylase activity of SIRT1 (10, 28). SIRT1 plays a role in beta cell insulin secretion and insulin sensitivity in other tissues such as fat and skeletal muscle(27). Importantly, SIRT1 expression is reduced in T2DM and is also down regulated by RAGE signaling (21, 50). Activation of RAGE signaling occurs via binding of its ligands such as AGEs. These RAGE ligands are known to be elevated in the T2DM condition, and have been related to insulin resistance (45). Specifically, exposure to the RAGE ligands reduces SIRT1 protein expression in the liver, skeletal muscle and adipose, resulting in the development of insulin resistance in these tissues (7).

In the current study, cRAGE correlated to GDR (r=0.343, *p*=0.003) (Table 4) and its reduction was strongly associated with the proportional odds for progression through the glucose tolerance continuum (Table 3). GDR is the gold standard measure for insulin-mediated glucose disposal which is largely dictated by the insulin sensitivity of the skeletal muscle. Therefore, failure of the cRAGE producing mechanisms such as RARβ, SIRT1, and ADAM10 in the skeletal muscle may allow for excessive RAGE signaling to promote the development of insulin resistance in skeletal muscle. This may

help explain why higher cRAGE is strongly related to insulin sensitivity and lower cRAGE is related to the progression toward T2DM.

Interestingly, we show that esRAGE is preferentially lost with obesity. We found a significant group effect of obesity, whereby Overweight-Obese individuals possessed a higher cRAGE:esRAGE ratio compared to Lean individuals suggesting a preferential loss of esRAGE. Although we did not see any difference in cRAGE:esRAGE when stratifying by obesity status alone, esRAGE was 35% lower in Obese compared to Lean individuals whereas cRAGE was 24% lower in obese compared to Lean individuals. In addition, the Overweight-Obese, IGT-T2DM group resulted in the lowest concentration of esRAGE (Figure 2). Both BMI and body fat percentage displayed stronger correlations with esRAGE compared to cRAGE (Table 4).

Production of esRAGE is regulated by the activity of two antagonistic splicing factors, heterogeneous nuclear ribonuclear protein A1 (hnRNPA1) and transformer-2 beta (TRA2β) (29). TRA2β promotes esRAGE production whereas hnRNPA1 suppresses this activity. Both TRA2β and hnRNPA1 are regulated by MAPK activity (1, 6, 51) which is well known to be activated by RAGE signaling and exacerbated in obesity and insulin resistance (24). In addition, RAGE expression plays a critical role in adipose differentiation, hypertrophy, and inflammation (15, 33, 44). Therefore, adipose expansion and subsequent adipokine mediated inflammation may be suppressing the splicing mechanisms that regulate esRAGE.

In support of this notion, TRA2β is reduced in the liver and skeletal muscle of obese, IGT-T2DM individuals (29, 35). In the current study, we found lower concentrations of esRAGE in obese individuals compared to lean. Additionally, esRAGE

was correlated to GDR (r=0.594, p<0.001), and body fat percentage (r=-0.311, p<0.001). These data suggest that both adipose, and skeletal muscle may be involved in RAGE splicing, and that the mechanisms involved become dysfunctional with obesity and insulin resistance. However, future studies are needed to identify the tissue- or, cell-specific sources of sRAGE isoform production, and what mechanisms are responsible for promoting and attenuating their release into the circulation.

These findings demonstrate that the study of sRAGE isoforms remains an important area of research given both old and new data (30) reporting the potential role of sRAGE to impart physiological benefit and protection from cardiovascular and metabolic disease. It is evident that the mechanisms of sRAGE production are tightly regulated and that relatively small changes in circulating concentrations are linked to the natural history of T2DM. Herein, we are the first to characterize the circulating concentrations of the two most prominent sRAGE isoforms across the glucose tolerance continuum and demonstrate that total sRAGE, cRAGE, and cRAGE:esRAGE were associated with the proportional odds for progression across the glucose tolerance continuum using ordinal logistic regression. Our data are admittedly, limited by not being age-matched across all groups, as others have demonstrated that chronological age plays a significant role in sRAGE concentrations (36). However, juxtaposition of the T2DM phenotype against a young, lean healthy phenotype, demonstrates the degree by which circulating sRAGE isoforms, in obesity, states of impaired glucose tolerance, and advanced age deviate from optimum health. To tease the effect of age away from these other factors, we compared circulating sRAGE isoform concentrations across GTS while covarying for age. This analysis revealed that sRAGE remained significantly reduced in

T2DM despite the age difference between the T2DM and LHC groups. However, covarying for age did eliminate differences in total sRAGE and cRAGE between NGT and IGT, as well as eliminate all differences previously observed for esRAGE and cRAGE:esRAGE. This in addition to the inverse correlations between sRAGE measures and age, implicate age to effect circulating sRAGE. However, given that differences were still realized between T2DM and LHC individuals indicates that the T2DM phenotype, regardless of age, is characterized in part by reduced total sRAGE and cRAGE.

We also acknowledge that the limitations of stratifying our data by BMI are such that BMI is less sensitive in detecting obesity than body fat percentage (38). However, when we stratified by body fat percentage cutoffs as previously reported by Romero-Corral et al, the findings were consistent with the data that is currently reported using BMI (38).

We were also unable to genotype our participants due to limited sample. This would have been an interesting addition to our data since multiple single nucleotide polymorphisms have been identified for RAGE and have been implicated in the development of obesity, and inflammation(23, 26). The SNP that involves glycine-serine switch at codon 82 (G82S) occurs in the ligand binding domain of RAGE and enhances its ability to promote RAGE activation (20). Koy et al demonstrated that lean and obese individuals with the S/S genotype possessed lower sRAGE compared to those with the G/S and G/G genotypes (26). Obese individuals in this cohort with the S/S genotype also possessed higher BMI and greater circulating CRP compared to those with the G/S and G/G genotypes (26). Our data demonstrate lower sRAGE isoform concentrations in

obese and T2DM individuals compared to lean healthy individuals. Unfortunately, we do not know if the G82S SNP is partly responsible for these differences in our sample on sRAGE. However, the frequency of glycine and serine alleles has not been previously shown to be different in T2DM compared to healthy individuals (23). Nevertheless, future studies should examine the effect of RAGE SNPs on the risk of obesity and diabetes development, and if this risk is related to sRAGE concentrations. The mechanism of how sRAGE concentrations are altered by SNPs in RAGE is also unclear and warrant future study. In addition, Kim OY et al only examined the relationship between the G82S SNP and total sRAGE concentrations and did not discriminate between the esRAGE and cRAGE isoforms (26). We have demonstrated here that dysregulation of these isoforms are associated with different phenotypes and therefore are likely under parallel regulation.

In conclusion, the disproportionate reductions of cRAGE and esRAGE in T2DM and obesity, respectively, require further mechanistic study as our data implicate adiposity and insulin sensitivity, or both, to play a role in sRAGE biology. These finding suggest the presence of a failure in the sRAGE producing mechanisms with the onset of T2DM and obesity and requires further study. sRAGE was also strongly associated with the proportional odds ratio for progression through the glucose tolerance continuum, asserting sRAGE as a potential biomarker for T2DM. The long-term benefits for reporting these data are: 1) to help direct research efforts toward elucidating failed mechanisms underpinning the discrepancy in sRAGE isoform expression in T2DM and obesity and 2) to determine the efficacy of targeting these mechanisms for treatment of T2DM and obesity.

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Author Contributions

ERM conceived of and designed the study, acquired and analyzed data, and drafted and reviewed the manuscript. VSS, JTM, BKB, SF, KK, CEF acquired and analyzed data and reviewed the manuscript. EW conceived of and designed the study, analyzed

data, and reviewed the manuscript. SRK acquired and analyzed data, reviewed the manuscript, obtained funding and supervised the study. JPK, LQ, and TPJS acquired and analyzed data, drafted and reviewed the manuscript, obtained funding and supervised the study. JMH conceived of and designed the study, acquired and analyzed data, drafted and reviewed the manuscript, obtained funding and supervised the study. JMH is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Figure and Table Legends

Figure 1 Soluble RAGE Isoforms According to Glucose Tolerance Status.
Subjects were stratified by glucose tolerance status NGT (n = 150): Normal Glucose Tolerance, IGT (n = 30): Impaired Glucose Tolerance, T2DM (n = 94): Type Two Diabetes Mellitus. Comparisons between groups were made for total sRAGE (A), cRAGE (B), esRAGE (C) and cRAGE: esRAGE ratio (D). Differences between groups were analyzed by one-way ANOVA and Bonferroni post hoc tests as necessary. Bars represent MEAN (SD). *p < 0.05, **p < 0.01, and ***p < 0.0001 vs. NGT.

Figure 2 Effects of Glucose Tolerance and BMI on sRAGE isoforms.
Subject groups were collapsed into NGT vs. IGT-T2DM designations and further stratified by BMI (Lean vs. Overweight-Obese). Lean, NGT n = 74; Overweight-Obese, NGT n = 76; Lean, IGT-T2DM n = 16; Overweight-Obese, IGT-T2DM n = 105. Group comparisons were made for total sRAGE (A), cRAGE (B), esRAGE (C), and cRAGE: esRAGE ratio (D) using two-way ANOVA and Bonferroni post hoc tests as necessary. Bars represent MEAN (SD). *p < 0.05, **p < 0.01, and ***p < 0.0001 vs. NGT; #p < 0.05, ##p < 0.01, and ###p < 0.0001 vs. Lean.

Table 1 Metabolic characteristics.
Data are presented as MEAN (SD). Normally distributed data were analyzed by one-way ANOVA and Bonferroni adjustments for multiple comparisons. Non-normally distributed variables (as indicated by ^) were analyzed using Kruskal-Wallis test and Bonferroni adjustments for multiple comparisons. NGT (n = 150): Normal Glucose Tolerance, IGT (n = 30): Impaired Glucose Tolerance, T2DM (n = 94): Type Two Diabetes Mellitus. BMI: body mass index; VO_{2Max}: Maximal Aerobic Fitness; BF%: Body Fat Percentage; Fat mass; 2-h OGTT Glucose iAUC: 2 Hour Oral Glucose Tolerance Test Glucose Incremental Area Under the Curve; 2-h OGTT Glucose: Blood glucose at 2-h time point of OGTT; HbA_{1c} : Glycated Hemoglobin; HOMA-IR: Homeostatic Model

Assessment of Insulin Resistance; GDR: Hyperinsulinemic-Euglycemic Clamp Derived Glucose Disposal Rate; hs-CRP: High Sensitivity C-Reactive Protein. $^{**}p < 0.01$, and $^{***}p < 0.0001$ vs. NGT; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.0001$ vs. IGT.

Table 2 Descriptive Demographics.
Frequencies of demographic descriptors of individuals grouped by glucose tolerance status. $^{***}p$ <0.0001 vs NGT.

Table 3 Soluble RAGE isoforms and proportional odds for developing T2DM.
Total sRAGE, cRAGE, esRAGE and sRAGE Ratio (cRAGE: esRAGE) were used to construct models 1, 2, 3 and 4 respectively. The values for total sRAGE, cRAGE and esRAGE were multiplied by 100 before entering them into the models. Models were corrected for age and race where Caucasian and lean were used as reference, respectively. OR: Odds Ratio, CI: confidence interval, Other: Hispanic/Asian

Table 4 Correlations Between sRAGE Isoforms and Metabolic Characteristics.
Bivariate correlation analyses were used to examine relationships between sRAGE isoforms and metabolic parameters. Pearson correlation coefficients were performed unless denoted (^) which were analyzed by Spearman's Rho.

826 **Table 1 Metabolic characteristics.**

Variable, units	NGT	IGT	T2DM
Sex, M/F	79/71	10/20	47/47
Age, y	39 (SD 17)	61 ± (SD 10)***	57 ± (SD 9)***
BMI, kg/m^2	27.0 (SD 6.2)	34.8 (SD 4.8)***	32.6 ± (SD 7.3)***
VO_{2Max}, mL/kg/min	32.6 (SD 10.4)	23.3 (SD 6.4)***	26.3 (SD 6.6)***
BF, %	33.0 (SD 9.4)	43.2 ± (SD 8.1)***	36.0 (SD 9.5)$^{\#\#}$
Fat Mass, kg	29.0 (SD 12.9)	40.7 (SD 8.5)***	31.7 (SD 12.4)$^{\#\#}$
Lean Body Mass, kg	55.3 (SD 12.1)	54.2 (SD 12.0)	57.9 (SD 11.5)
2-h OGTT Glucose, mg/dL	114 (SD 22.5)	162 (SD 16.9)***	281 (SD 67.4)***$^{\#\#\#}$
2-h OGTT Glucose iAUC, AU	4201 (SD 2083)	7322 (SD 2639)**	5133 (SD 5567)$^{\#}$
HbA1C, %	5.4 (SD 0.46)	5.7 (SD 0.52)	7.1 (SD 1.6)***$^{\#\#\#}$
HbA1C, mmol/mol	35.7 (SD 4.98)	38.5 (SD 5.71)	54.5 (SD 17.7)***$^{\#\#\#}$
Fasting Glucose, mg/dL^	93 (SD 10.9)	97 (SD 11.7)	151 (SD 61.4)***$^{\#\#\#}$
Fasting Insulin, mU/L^	9.5 (SD 6.3)	15.9 (SD 11.6)**	13.5 (SD 6.6)***
HOMA-IR, AU^	2.2 (SD 1.6)	4.7 (SD 5.0)***	5.0 (SD 3.2)***
Matsuda Index, AU^	4.7 (SD 3.1)	2.5 (SD 1.5)***	3.1 (SD 1.9)***
GDR, mg/kg/min^	4.9 (SD 2.3)	2.9 (SD 1.2)**	2.6 (SD 0.96)**
hs-CRP, mg/L	2.2 (SD 1.9)	2.8 (SD 1.6)	2.6 (SD 2.6)

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Table 2 Descriptive Demographics.

	NGT (n=150)		IGT (n=30)		T2DM (n=94)	
Variable	**n**	**%**	**n**	**%**	**n**	**%**
Age (y)	39 (SD 17)		61 (SD 10)***		57 (SD 9)***	
Young (18-35 y)	88	59	1	3	1	1
Middle age (36-64 y)	44	29	19	63	68	72
Old (≥ 65 y)	18	12	10	33	25	27
Gender						
Male	79	53	10	35	47	50
Female	71	47	20	67	47	50
Race						
White	107	71	21	70	63	67
Black	17	11	7	23	31	33
Hispanic	10	7	2	7	0	0
Asian	16	11	0	0	0	0
Obesity (kg/m^2)	27.0 (SD 6.2)		34.8 (SD 4.8)*		32.6 (SD 7.3)*	
Lean (18-24)	74	49	1	3	15	16
Overweight (25-29)	27	18	2	7	24	26
Obese (≥ 30)	49	33	27	90	55	59

Table 3 Soluble RAGE isoforms and proportional odds for developing T2DM.

	Model 1			Model 2			Model 3			Model 4		
Variable	***OR***	***95% CI***	***P***	***OR***	***95% CI***	***P***	***OR***	***95% CI***	***P***	***OR***	***95% CI***	***P***
Age	1.06	1.03-1.08	<.001	1.06	1.03-1.08	<.001	1.07	1.05-1.10	<.001	1.06	1.04-1.09	<.001
Race												
Black	3.43	1.69-6.96	<.001	4.11	1.93-8.75	<.001	3.57	1.74-7.31	<.001	4.04	1.91-8.53	<.001
Other (Hispanic/Asian)	0.30	0.06-6.96	0.139	0.31	0.06-1.55	0.155	0.36	0.07-1.72	0.199	0.40	0.08-1.93	0.255
Obesity												
Overweight	1.39	0.58-3.35	0.459	1.58	0.64-3.87	0.322	1.29	0.53-3.16	0.576	1.79	0.72-4.45	0.208
Obese	1.08	0.46-2.50	0.864	1.34	0.57-3.15	0.505	1.06	0.45-2.53	0.890	1.68	0.69-4.07	0.253
Total sRAGE	**0.91**	**0.85-0.97**	**0.003**	-	-	-	-	-	-	-	-	-
cRAGE	-	-	-	**0.84**	**0.77-0.92**	**<.001**				-	-	-
esRAGE	-	-	-				**0.93**	**0.78-1.10**	**0.374**	-	-	-
cRAGE/esRAGE	-	-	-	-	-	-	-	-	-	**0.74**	**0.58-0.96**	**0.022**
C-statistics		**0.782**			**0.805**			**0.773**			**0.784**	

Table 4 Correlations Between sRAGE Isoforms and Metabolic Characteristics.

	Total sRAGE (pg/ml)		cRAGE (pg/mL)		esRAGE (pg/mL)		cRAGE:esRAGE	
	r	***p***	***r***	***p***	***r***	***p***	***r***	***p***
Age (y)	-0.368	< 0.001	-0.387	< 0.001	-0.206	0.001	-0.254	<0.0001
VO_{2Max} (mL/kg/min)	0.231	0.002	0.291	< 0.001	0.156	0.039	0.202	0.007
BMI (kg/m^2)	-0.225	< 0.001	-0.158	0.010	-0.288	< 0.001	0.140	0.023
BF (%)	-0.288	< 0.001	-0.227	0.001	-0.311	< 0.001	-0.004	0.953
LBM (kg)	0.066	0.351	0.075	0.297	-0.058	0.414	0.136	0.058
Fat Mass (kg)	-0.211	0.003	-0.130	0.071	-0.312	< 0.001	0.101	0.158
2-h OGTT (mg/dL)	-0.233	0.002	-0.292	< 0.001	-0.075	0.332	-0.253	0.001
2-h OGTT iAUC (AU)	-0.068	0.185	0.078	0.300	-0.279	< 0.001	0.424	< 0.001
HbA1C (%)	-0.200	0.006	-0.183	0.013	-0.153	0.036	-0.001	0.989
FPG (mg/dL)	-0.292	< 0.001	-0.337	< 0.001	-0.134	0.046	-0.233	< 0.001
FPI (mU/L)	-0.184	0.006	-0.200	0.003	-0.107	0.116	-0.068	0.322
HOMA-IR (AU)	-0.255	< 0.001	-0.291	< 0.001	-0.121	0.075	-0.154	0.024
Matsuda Index (AU)	0.214	0.005	0.183	0.018	0.187	0.015	-0.007	0.928
GDR (mg/kg/min)	0.472	< 0.001	0.343	0.003	0.594	< 0.001	-0.276	0.018
CRP (mg/L)	-0.220	0.012	-0.138	0.119	-0.274	0.002	0.140	0.113

Figure 1 Soluble RAGE Isoforms According to Glucose Tolerance Status.

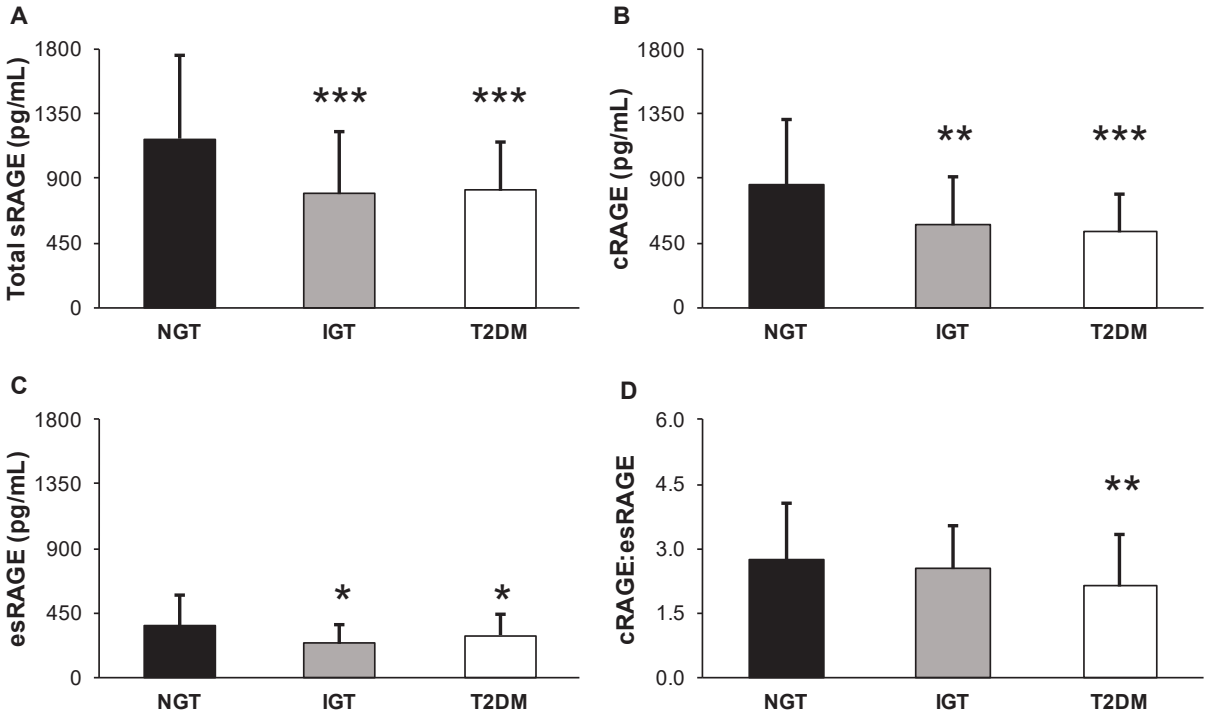

Figure 2 Effects of Glucose Tolerance and BMI on sRAGE isoforms.